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# Advances in flow cytometry in basic and applied equine andrology

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## Abstract

The aim of this review is to present the current probes available that assess different compartments and functions of stallion spermatozoa, including assays to investigate the functionality of the membranes, nucleus and mitochondria, and to study cell signaling in this particular cell. New multi-parametric protocols for the assessment of stallion sperm, recently developed in the laboratory of the authors, will also be presented. The potential clinical applicability of diagnostic tests based on flow cytometry will also be discussed.

**Keywords**: fluorescent probes, sperm, stallion.

## Introduction

Particularly the last decade of the current century, has been witness to intensive research in sperm biology. Consequently, a better understanding of sperm function in relation to clinical andrology and sperm biotechnologies (Peña *et al*., 2011, 2015) has arisen. Relevant advances in stallion sperm biology include, among others, the following: the understanding of osmotic shock (Ball and Vo, 2001; Pommer *et al*., 2002; Ball, 2008) and its implications on cryopreservation, which promoted the development of new protocols based on more permeant cryoprotectants (Oldenhof *et al*., 2010, 2012, 2013; Hoffmann *et al*., 2011; Pukazhenthi *et al*., 2014); advances in understanding the role of reactive oxygen species (ROS; Gibb *et al*., 2014, 2015; Varner *et al*., 2015; Gibb and Aitken, 2016); and the development of practical methods for stallion sperm separation and selection through colloidal centrifugation (Waite *et al*., 2008; Johannisson *et al*., 2009; Morrell *et al*., 2009a, b; Edmond *et al*., 2012; Crespo *et al*., 2013; Ponthier *et al*., 2013). More recently, the understanding of sperm bioenergetics and mitochondrial functionality have become two hot topics in stallion andrology. Beside these advances, better tools for sperm assessment have been developed, in which flow cytometry has played a major role. The aim of this review is to present a rapid summary of the current probes available to assess stallion sperm and describe new protocols for the assessment of stallion sperm, including those recently developed in the laboratory of the authors. The potential clinical applicability of a diagnostic test based on flow cytometry will also be discussed. Interestingly, these assays have been recently supported with field fertility data (Barrier Battut *et al*., 2016).

## Basic principles of flow cytometry applied to sperm analysis

Flow cytometry measures multiple parameters of cells that rapidly flow in a stream through a system of photonic receptors. The properties measured include the size of the spermatozoa in the forward scatter detector (FSC), the complexity in the side scatter detector (SSC) and the relative fluorescence intensity in fluorescence detectors (FL). These characteristics are detected using a fluidic and optical to electronic coupling system that records how each individual spermatozoon or other particle presents in the sample, scatters incident laser light, and emits fluorescence. In the flow cytometer, spermatozoa are carried to the laser interrogation point in a fluid stream (sheath fluid). When they pass through the laser intercept, they scatter laser light, and any fluorescent molecules present are excited and emit light in different wavelengths. Appropriately positioned lenses collect the scatter and fluorescent light. A combination of beam splitters and filters steer fluorescence to detectors that produce electronic signals proportional to the optical signals striking them. List mode data are collected on every single spermatozoon and stored in the computer; these data are analyzed and provide information about subpopulations in the sample and are displayed graphically in histograms and dot plots. Fluorescent compounds are used to study stallion sperm functionality. A fluorescent compound absorbs light energy over a range of wavelengths characteristic for each. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level; the electron quickly returns to the ground state, releasing the excess energy as a photon. This transition of the energy is termed fluorescence. The range of wavelengths in which a fluorescent compound can be excited is called absorption spectrum, while the range of wavelengths emitted is called emission spectrum. Ideally, the light produced by emission should be different from the light used for excitation, and this difference is known as the *Stokes Shift*. The wavelength of emission is longer than the wavelength of

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excitation because typically more energy is used to excite the electrons of the fluorochrome than the energy released (as light) when the electrons return to the resting state. For example, a commonly used fluorochrome, fluorescein isothiocianate (FICT), absorbs light in the range 400-550 nm, with a peak or maximum excitation at 490 nm (the laser is used to excite a particular dye, the blue laser (488 nm) in this case), and emits in the range 475-700 nm, with a peak at 525 nm (green spectrum). This range of wavelengths determines the filters and the channels (fluorescence channels FL) of detection to be used. Combining different flourochromes with multiple wavelengths of excitation and emission allows multiple and simultaneous measurements, however compensation for spectral overlap has to be considered and carefully managed. When two or more dyes are used simultaneously, there is a chance that their emission profiles will coincide, making measurement of the true fluorescence for each one difficult. This outcome can be avoided by using dyes at distant positions in the spectrum; for example, a dye that is excited with the violet laser (405 nm) and a dye excited with the red laser (647 nm). However, using dyes at distant positions is not always possible, and a process called fluorescence compensation is applied. This process calculates how much interference, as a percentage, a fluorochrome will have in a channel that was not assigned specifically to measure it. The design of an experiment in the flow cytometer implies careful selection of probes suitable for each particular cytometer, identification of potential spectral overlap among probes, use of proper controls for positive and negative populations (unstained sample), and controls for compensation (single stained samples in which there is a stained and unstained population for each dye to be used in the experiment). Depending on each particular experiment, other controls can be necessary, including fluorescence minus one (FMO) controls, isotype controls or secondary antibody only controls.

## The sperm membrane: integrity, permeability, fluidity, and functionality

Traditional assessment of the sperm membrane has focused on the physical integrity using dye exclusion tests. Classical combinations of fluorescent probes for this purpose include the combination of SYBR-14 and propidium iodide (PI). This combination of probes requires the blue laser for excitation (488 nm) and provides two wavelengths of emission; green for live sperm (521 nm SYBR-14) and red for dead sperm (635 nm PI). This combination of probes allows the rapid discrimination of debris (because both are DNA binding probes), and both probes are excited with the blue laser (488 nm). Spillover between emission wavelengths of both probes can occur (521 nm for SYBR-14 and 635 nm for PI), and proper fluorescence compensation has to be established in order to use this probe pair because SYBR-14 still has, on average, a 6% emission at 635 nm. Furthermore, staining with SYBR-14/PI discriminates only between live and dead sperm and does not expose initial states of membrane damage. Additionally, doublets have to be identified to correctly interpret this assay. Alternatively, Hoechst 33342 and PI (Plaza Davila *et al*., 2015) also allow the rapid discrimination of debris and have the advantage that spillover is unlikely due to the distinct excitation and emission spectra of these probes. However, H33342 needs a violet or ultraviolet laser for excitation. Detection of more subtle changes in the sperm membrane requires the use of other probes. Fluidity of sperm membranes can be assessed with merocyanine 540 (da Silva *et al*., 2011), and subtle increases in the permeability of the plasma membrane can be detected with YoPro-1 (Gallardo Bolanos *et al*., 2012, 2014a). Yo Pro-1 is routinely used in the authors' laboratory in combination with PI; H33342 is also incorporated to sort debris. These combinations allow the detection of changes in sperm membranes at much earlier stages than SYBR-14 and correlate better with motility and sperm velocities (Gallardo Bolanos *et al*., 2012).

Recently, new fixable fluorescent dyes have become available in multiple colors, which facilitate experiments with multiple spectra in fixed sperm. These probes are based upon the reaction of fluorescent reactive dye with cellular amines. These are proprietary dyes that can permeate the compromised membranes of necrotic cells and react with free amines both in the cytoplasm and on the cell surface, resulting in intense fluorescent staining. In contrast, only the cell surface amines of intact cells are available to react with the dye, resulting in relatively dim staining. The discrimination is maintained following formalin-fixation of the sample under conditions that inactivate pathogens. Moreover, these assays use only one channel of the flow cytometer, leaving the other channels available for multicolor panels. The potential advantage of these dyes is the ability to process and stain the samples at locations remote to the flow cytometer.

The evaluation of the sperm's ability to undergo the acrosome reaction in response to an agonist challenge, is useful in cases of infertility in certain thoroughbred lines. This assay, the Acrosomal Responsiveness Assay (ARA; Johnson *et al*., 2008; Vaner, 2008), evaluates the ability of the acrosome to react when challenged with the $Ca^{2+}$ ionophore, A23197. Common probes to assess acrosomal integrity, either in a basal status after a challenge, are those which recognize targets inside the acrosome, including specific lectins (*Pisum sativum* agglutinin PSA, and *Arachis hypogea* agglutinin PNA) that bind to glucosidic residues in different parts of the acrosomal membrane. The acrosome has also been monitored in human sperm with anti-CD46 antibodies (Carver-Ward *et al*., 1994; Grunewald *et al*., 2008).

**Mitochondria and stallion sperm functionality**

The mitochondria of spermatozoa are increasingly studied in both basic and applied andrology (Gibb *et al*., 2014; Peña *et al*., 2015; Plaza Davila *et al*., 2015). Stallion spermatozoa are highly dependent on mitochondrial production of ATP, and mitochondrial malfunction leads rapidly to sperm senescence and death. Stallion spermatozoa have particularly active mitochondria, and as a result, they generate large amounts of reactive oxygen species (ROS; Gibb *et al*., 2014; Plaza Davila *et al*., 2015). Sperm mitochondria are sensitive indicators of sperm stress during processes such as cooling and cryopreservation (Ortega-Ferrusola *et al*., 2008, 2009a). Two common probes are used to assess stallion mitochondrial function by flow cytometry. The probe 5,5', 6,6'–tetrachloro-1,1', 3,3' tetraethylbenzymidazolyl carbocianyne iodide (JC-1) forms multimeric aggregates in mitochondria with high membrane potential (active mitochondria). These aggregates emit the high orange wavelength of 590 nm when excited at 488 nm. In mitochondria with low membrane potential (inactive mitochondria), JC-1 forms monomers that emit in the green wavelength (525 to 530 nm) when excited at 488 nm (Garner and Thomas, 1999; Gravance *et al*., 2000). Recently, mitotracker dyes (Gallon *et al*., 2006; Sousa *et al*., 2011) have become available in multiple colors and provide colorful alternatives to be used in multicolor experiments. Both JC-1 and Mitotracker deep red have been recently used in our laboratory (Gallardo Bolanos *et al*., 2014a). These probes measure different aspects of mitochondrial function; JC-1 reflects mitochondrial membrane potential, while mitotracker deep red passively diffuses across membranes and binds to thiols in active mitochondria (Peña *et al*., 2016).

**The sperm DNA**

The sperm chromatin structure assay (SCSA) has been extensively used. In stallion andrology, this assay has successfully discriminated between stallions of low, high, and average fertility (Love and Kenney, 1998; Love, 2005). In spite of the importance of DNA, the origin of damage to sperm DNA is still largely ignored. In human andrology, it is becoming clear that two major factors are associated with damage to sperm DNA: oxidative stress and protamination of the spermatozoa. These two features are strongly linked because defective protamination renders spermatozoa more susceptible to oxidative damage (Aitken and De Iuliis, 2010; Aitken *et al*., 2013, 2014; Gavriliouk and Aitken, 2015). Recent research from our laboratory shows evidence indicating that DNA damage in stallion spermatozoa is oxidative as well (Balao da Silva *et al*., 2014). Oxidative stress can be assessed using specific antibodies against the oxidized form of guanine; 8-oxoguanine in fixed, permeabilized samples (Balao da Silva *et al*., 2016).

**The stallion sperm: a redox regulated cell**

Reactive oxygen species (ROS) as by-products of various metabolic processes may have detrimental effects, but ROS may also be important regulators of cellular functions (Stowe and Camara, 2009). These are chemical species formed after incomplete reduction of oxygen and include the superoxide anion ($O_2\bullet^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical (OH•). Superoxide anion ($O_2\bullet^-$) can be generated at different points within the mitochondrial electron transport chain (ETC) by univalent reduction of oxygen. Most superoxide is converted to $H_2O_2$ by superoxide dismutase inside and outside of the mitochondrial matrix, and superoxide in low and controlled amounts exerts important regulatory cellular functions. Excessive $H_2O_2$ can combine with $Fe^{2+}$ to form reactive hydroxyl radical (OH•; Shen *et al*., 1992). Superoxide is short lived ($t_{1/2}$ = 1 ms) and cell impermeable, while $H_2O_2$ is more stable and cell permeable. In the presence of nitric oxide (NO•), $O_2\bullet$- forms the reactant peroxynitrite (ONOO•), and ONOOH induced nitrosylation of proteins, DNA, and lipids can modify their structure and function (Stowe and Camara, 2009). NO• is synthesized through the conversion of l-arginine to l-citruline by nitric oxide synthase (NOS). These enzymes are present in stallion spermatozoa, possibly as sperm specific isoforms (Ortega-Ferrusola *et al*., 2009b). Numerous studies indicate that ROS are important regulators of sperm function (Zini *et al*., 1995; Aitken *et al*., 1997; de Lamirande and Gagnon, 2002, 2003; De Lamirande and Lamothe, 2009), and ROS become detrimental only if homeostasis is lost (Peña *et al*., 2015). Moreover, recent evidence suggests that stallion sperm mitochondria produce significant amounts of NO (Ortega-Ferrusola *et al*., 2009b). Nitric oxide has a relatively long half-life (1 s) and is more reactive than $O_2\bullet^-$. Controlled ROS production occurs during capacitation in spermatozoa (Agarwal *et al*., 2014). This controlled production triggers signaling pathways initiated by an increase in cyclic adenosine 3'-5' monophosphate (cAMP). Increased cAMP activates protein kinase A (PKA), and the subsequent phosphorylation of extracellular regulated -kinase-like proteins and finally tyrosine phosphorylation of proteins in the fibrous sheath of the spermatozoa, leading to sperm hyperactivation. Numerous assays have been developed to assess oxidative stress and production of ROS in the stallion spermatozoa (Baumber *et al*., 2002; Sabeur and Ball, 2006; Burnaugh *et al*., 2007; Gibb *et al*., 2014; Plaza Davila *et al*., 2015). Flow cytometry can be used to detect specific reactive oxygen species (ROS), reactive nitrogen species (RNS) and the consequences of perturbed ROS homeostasis, such as lipid peroxidation, DNA oxidation, increased membrane permeability and protein oxidation. Reactive oxygen species can be

detected using different probes. The superoxide indicator dihydroethidium, also called hydroethidine, exhibits blue-fluorescence in the cytosol until oxidized, where it intercalates within the cell's DNA, staining its nucleus a bright fluorescent red. Mitosox Red™ is used to specifically detect mitochondrial $O_2\bullet^-$. Hydrogen peroxide can be detected using dichlorodihydrofluoresceindiacetate, although this probe is not highly specific for $H_2O_2$. Recently, molecules such as aryl boronate have been described and appear highly specific for the detection of $H_2O_2$ in spermatozoa (Purdey *et al*., 2015). Other probes have been recently introduced. The cellrox sensors™ are available in different colors. These may be fixed after staining, facilitating their use in multicolor panels. In our laboratory, the CellROX deep red ™has been used in multicolor experiments, especially to detect mitochondrial $O_2\bullet^-$ and the hydroxyl radical (OH•; Gallardo Bolanos *et al*., 2014b; Plaza Davila *et al*., 2015). It is extremely important to consider the conditions in which the assay is performed. High Cell ROX deep red™ fluorescence may indicate either mitochondrial activity or real oxidative stress. The particular dependence of stallion spermatozoa on oxidative phosphorylation to generate ATP (Plaza Davila *et al*., 2015) may reflect the apparent paradoxical relationship between ROS and sperm functionality due to increased electron leakage and thus increased $O_2\bullet^-$ production (Ortega-Ferrusola *et al*., 2010; Gibb *et al*., 2014; Yeste *et al*., 2015).

The oxidation of the plasma membrane leads to increased membrane permeability (Christova *et al*., 2004) that can be monitored with YoPro-1 (Ortega-Ferrusola *et al*., 2008; da Silva *et al*., 2011; Gallardo Bolanos *et al*., 2012; Garcia *et al*., 2012; Gibb *et al*., 2014). Oxidation of DNA can be monitored using antibodies against the oxidized form of guanine. Peroxidation of sperm membranes can be detected with the probe BODIPY® 581/591 C11. This probe emits orange-red fluorescence in the non-oxidized state, shifting to green florescence when peroxidized (Ball and Vo, 2002; Ortega-Ferrusola *et al*., 2009c). Lipid peroxidation is also monitored by detection of 4-hydroxynonenal (4-HNE) using specific antibodies, a product from the oxidation of sperm- membrane lipids (Aitken *et al*., 2012; Gibb *et al*., 2014). This assay is considered a reliable indicator of ROS imbalance, and specific protocols for stallion spermatozoa have been recently published (Martin Munoz *et al*., 2015).

## Sperm senescence

Senescent spermatozoa express active caspase 3 (Amann, 2010; Aitken and Baker, 2013; Aitken *et al*., 2015). Depending of the presence of pro-survival factors, caspase 3 remains inactive due to the phosphorylation of protein kinase B (PKB or Akt; Gallardo Bolanos *et al*., 2014a). If pro-survival factors are lost or oxidative stress reaches a threshold, caspase 3 is activated and sperm senescence and death are triggered (Gallardo Bolanos *et al*., 2014b). Cryopreservation triggers this phenomenon, and surviving spermatozoa experience accelerated senescence (Thomas *et al*., 2006; Ortega-Ferrusola *et al*., 2008, 2009a). Active caspase 3 can be detected using CellEvent™ Caspase-3 Green Detection Reagent, which consists of a four-amino-acid peptide (DEVD) conjugated to a nucleic acid-binding dye. This cell-permeant substrate is intrinsically non-fluorescent because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase-3 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with an absorption/emission maximum of ~502/530 nm.

## Concluding remarks

Flow cytometry is a powerful tool in andrology, allowing the rapid and simultaneous assessment of multiple sperm compartments and functions in thousands of spermatozoa in a few seconds. Recent data also suggests that the data generated are powerful forecasts of field fertility and an extremely important tool for quality control in stallion stations.

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